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"The Approach to Muscle Proteins with an Attention to Chemical Cross-linking"

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To explore the intrinsic property which resides in the interaction of muscle proteins, the cross-linking method was used. The cross-linking of filamentous actin with p-NN′-Phenylenebismaleimide forms oligomers of actin and the cross-linking of filamentous actin and myosin subfragment-1 (S1) with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide forms the complex of actin and S1 (Acto* S1). The former is the cross-linking within filaments, i.e. between monomers of the same long-pitched helix, and the latter is not within filaments, but between actin subunit and S1.

In this report, efforts were concentrated on the study to see if strain imposed partially on the filaments by cross-linking affects the whole strand. For this purpose both cross-linked products were depolymerized, purified and mixed with normal actin monomers and brought to the repolymerizing condition. Firstly, the depolymerization of the cross-linked actin produces oligomers. Oligomers accelerate the rate of the monomer polymerization.

Oligomers work as nuclei for polymerization. Whereas oligomers seem to have another function to enhance the network or bundle formation of actin filaments, since one of the results can not be ascribed to nuclei formation, that is, after adding of EGTA, oligomers enhanced only the rate of fluorescence anisotropy increase of actin filaments without affecting the rate of viscosity increase. The present conjecture of the network or bundle formation of actin filaments seems to explain the above results well.

Secondly, the depolymerization of the filamentous Acto* S1 bring the Mg-ATPase reduction. The loss of Mg-ATPase activity is recovered with mixing of actin monomers and inducing assembyl of monomers. Although it is preliminary observations in electron-micrographs, compared with the extent of Mg-ATPase recovery, the Acto*S1 reassembled into filaments are few. Instead, a number of round or short particles were observed. It appaears that the assembly of actin monomers in the presence of the depolymerized Acto*S1 complexes form short particles possesing the Mg-ATPase activity to some extent.

The K-EDTA ATPase activity of the depolymerized Acto*S1 complexes is also reduced and which is reactivated with addition of actin monomers and salt. In contrast, the K-EDTA ATPase activity of S1 is depressed by actin. These results suggest that the cross-linking introduces strain into the structure. Resasemblies of the Acto*S1 with actin monomers restore the original conformation of S1.

Summarizing these results, small amount of cross-linked species influence greately the conformation of actin filaments and also themselves are influenced by filaments.

KEY WORDS: Actin/ Myosin-subfragment 1/ Cross-linking/ Anisotropy/ Oligomer/

INTRODUCTION

The force of muscle contraction is generated by interaction of myosin and actin.

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Abbreviations: pPDM; p-NN′-Phenylenebismaleimide, EDC; 1-ethyl-3(3-dimethylaminopropyl) carbodiimide, EGTA; ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, AEDANS; N-(Iodoacetylaminoethyl)-5-naphtylamine-1-sulfonic acid PIA; N-(1-pyrenyl)iodoacetamide, SDS; sodium dodecylsulfate, PAGE; polyacrylamide gel electrophoresis

Thus elucidation of the mode of interaction is interesting not only from the point of view to understand the mechanism of muscle contration but also from the pure physico-chemical interest on the thermodynamics concerned with conformational transition of macromolecules. There we can expect to find a subtle molecular architecture to convert chemical energy to mechanical energy. In the present report the interactions of muscle proteins were studied with a method of chemical cross-linking. The cross-linking is useful to fix the fluctuation of molecules in a binding equilibrium, or to keep molecules in a close contact. Two types of cross-linker; pPDM and EDC were selected. The former joins the subunits of actin filaments[1]. Normally actin filaments (F-actin) depolymerize into subunits, i.e. actin monomers (G-actin) at low ionic strength. Instead, the cross-linked subunits from oligomers after depolymerization. This reagent cross-links a cystein residue on one subunit with a lysin residue on a neighboring subunit.[1] Whereas the latter was used for the cross-linking of actin filament with myosin subfragment 1 (S1). Mg-ATPase is activated until almost V_{max} by this carbodiimide zero-length cross-linking.[2] Amino groups of S1 and carboxy group of actin are considered to be responsible for this covalent binding. The aim of the work in this report is to see if molecular distortion subjected partly on the actin filaments modify the overall conformation of actin filaments. For this purpose the cross-linked actin filaments and the cross-linked Acto*S1 filaments were depolymerized successfully and after purification the cross-linked species were mixed with actin monomers and assemblies were induced. The strain in the cross-linked species is transferred locally into subunits in contact with them and then transmitted over the whole filament, which appears to cause the network or bundle formation, or the shape transformation from the filaments to the short irregular particles.

Nowadays, it is well understood that actin and myosin are distributed widely from animal to plant kingdom. The complicated and versatile propeties of actin and myosin were obtained through the evolution. To understand more clearly the characteristics of both proteins, the importance of the study from the evolutional point of view was emphasized.

EXPERIMENTAL

Materials: Actin and myosin were obtained from rabbit skeletal muscles according to the methods described previously.[4-5] S1 was obtained by digestion of myosin with chymotrypsin.

Labelling of actin: F-actin in 0.1 M KCl, 20 mM Tris-HCl (pH 8.0), 0.02 mM $CaCl_2$ and 1 mM NaN_3 was labelled with AEDANS in a ratio of 1/0.8 for 48 hr at 4°C. After centrifugation of these labeled F-actin at 80,000 g for 3 hr, the pellet of labelled F-actin was dialysed against 500 ml of A-buffer (0.2 mM $CaCl_2$, 0.2 mM ATP, 2 mM Tris-HCl (pH 8.0), and 1 mM NaN_3), for 2-days with two exchanges of dialysate. The resulting depolymerized actin (G-actin) was centrifuged at 140,000 g for 90 min and aggregated materials were removed by Sephacryl S-200 column (Pharmacia Co.).

Labeling of S1: S1 was labeled to monitor its concentration in the Acto*S1 complex. S1 in a concentration of 15 μM were labeled with PIA in a ratio of 1/1 in 30 mM NaCl, 10 mM Tri-HCl (pH 8.0) and 1 mM NaN_3. After labeling free dyes were removed by dialysis or G-25 coulmn with a use of same buffer.

Cross-linking of actin filament with pPDM: F-actin was cross-linked with a method of Knight and Offer (1978)[1] at 25°C for 1 hr, then depolymerized with dialysis against A-buffer at 4°C overnight. The cross-linked actin was applied to Sephacryl S-200 column (Pharmacia Co.) and the peak of the fraction was concentrated by immersible CX-10 (Millipore Corp.).

Cross-linking of F-actin and S1 by EDC: Cross-linking of actin and S1 was performed as described by Mornet et al[2]. The complex was separated from the free S1 in 10 mM ATP, 2 mM $MgCl_2$, 0.1 M KCl, 50 mM HEPES (pH 7.5) by centrifugation at 140,000 g for 45 min at 4°C, and depolymerized by dialysis against A-buffer overnight at 4°C. After centrifugation as before, the depolymerized complex was obtained in the supernatant. Then the crude Acto*S1 complex was applied to AcA 34 Ultrogel (LKB) column (2.5 cm $\times$ 74 cm) to remove free actin monomer. Using air fuge (Beckman), repolymerization or reassembly of the Acto*S1 was examined by centrifugation at 140,000 g for 30 min.

ATPase measurements: Mg-ATPase of the cross-linked complex was studied by pyruvate kinase and lactate dehydrogenase (LDH) system containing 25 mM $MgSO_4$, 25 mM KCl, 163 mM Tris-HCl (pH 7.5), 0.1 mM NADH, 6.8 mM phosphoenolpyruvate, 0.01 mM of pyruvate kinase and lactate dehydrogenase solution (Sigma No. 40–7) in total volume of 0.588 ml including 0.15 ml of sample (cross-linked complex) and 5 μl of ATP (final conc. 0.85 mM).
Samples were incuvated in 2 mM $MgCl_2$ for 30 min at 20°C after adding of G-actin. The amount of ADP liberated by ATPase was measured by monitoring the absorption decrease of NADH at 340 nm using the spectrophotometer (Gilford) at 25°C, and the rate of ATPase was obtained from the initial slope.

K-EDTA ATPase was measured according to Taussky and Shorr[6].

Static anisotropy of fluorescence: Static anisotropy of AEDANS-actin was measured according to Ref. 7.

Viscosity: Viscosity was measured with Ostwald viscometer.

Gel electrophoresis: SDS-PAGE was performed according to Ref. 8.

Equipments: Fluorescence was measured with Jobin-Yvon spectrofluorimeter and absorbance was measured with spectrophotometer (Gilford Co.).

RESULTS

1. The characteristics of actin oligomers obtained by pPDM cross-linking

1.1 The nuclei formation by oligomers on the polymerization of G-actin

The depolymerization of cross-linked filaments produces oligomers. From the crude solution of oligomers, free G-actin were eliminated by column chromatography (Fig. 1). According to PAGE, the first and second fraction contains oligomers and monomers respectively. Oligomers consist mainly of dimers and trimers. When

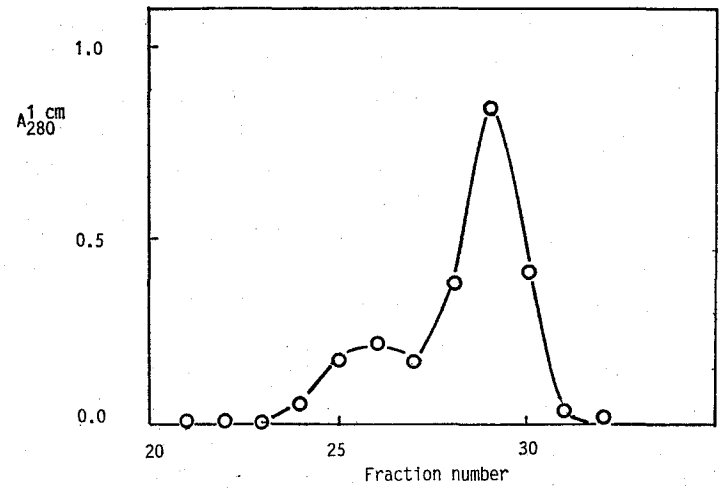

Fig. 1. Column chromatography of actin oligomers obtained by depolymerization of F-actin cross-linked with pPDM. 8 ml of depolymerized actin (2.6 mg/ml) was applied to the Sephacryl S–200 (Pharmacia Co.) column; 2.6cm (in dia.) ×80 cm (in length). Elution rate was 18 ml/hr, and 6 ml/tube was collected. Fraction 25 and 26 were combined and concentrated by Immersible CX–10 (Millipore Corp.)

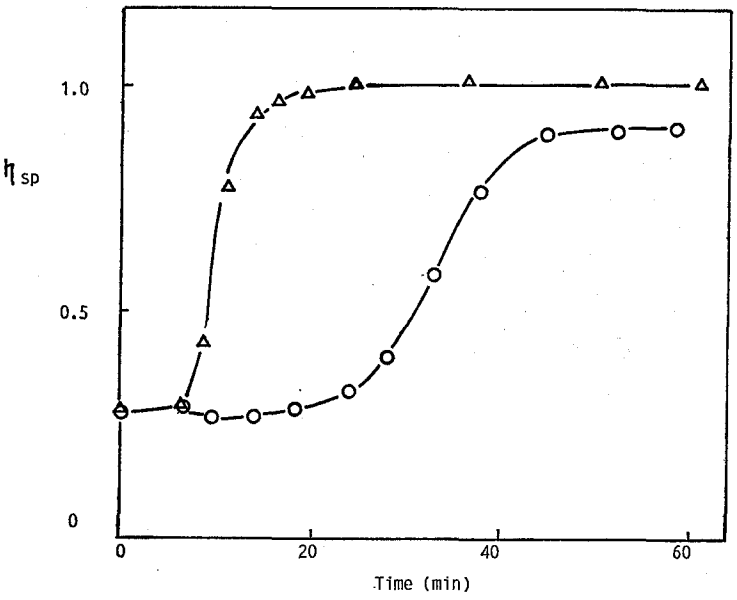

Fig. 2. Effects of oligomers on the rate of actin polymerization studied by viscometry. The polymerization of AEDANS-actin monomers (0.49 mg/ml) in A-buffer was induced in 0.1 M KCl in the presence and absence of oligomers. To compare the result with the fluorescence anisotropy measurements, AEDANS-actin monomers were used and in order to balance the concentration of the control with that of the sample in the presence of oligomers, 0.034 mg/ml of non-labeled actin monomer were added to the control. (○); control, (△); 0.034 mg/ml oligomers

KCl is added, G-actin in low ionic strength polymerize and form long filaments. This polymerizations are observed by the increase in viscosity and in static anisotropy of flourescence. For the anisotropy measurement, actin was labeled with a fluorescent probe; AEDANS. Actin polymerization is a condensation phenomenon[9-10]. At first, the polymerization of actin monomers was observed by viscometer. Oligomers were added to G-actin and polymerization was induced. As seen in Fig. 2, oligomers accelerate the polymerization of G-actin. Oligomers act as nuclei and the numbers of filaments polymerized increase compared with those in their absence. This induces the acceleration of the rate of viscosity increase. Finally, very long filaments are formed. With light scattering methods, Ooi[11] (1960) measured the average molecular weight of filaments (about 8,000,000).

Next polymerization was followed by static anisotropy of fluorescence. Varying the amount of oligomers added to AEDANS-actin monomers, the change of static anisotropy was measured (Fig. 3). The static anisotropy of AEDANS-actin increased from around 0.15 at the monomer state to 0.25 at the filamentous state. This increase in the static anisotropy is due to the restriction of the rotation of the AEDANS-actin monomers as a whole in the filaments.[7] Oligomers accelerate the increase in the static anisotropy of AEDANS-actin. The more the amount of oligomers added, the higher the rate of anisotropy increase, although the levels of anisotropy attained were almost identical. Consistently with the results of viscosity, static anisotropy of fluorescence also shows the increase of the number of filaments formed in the presence of oligomers.

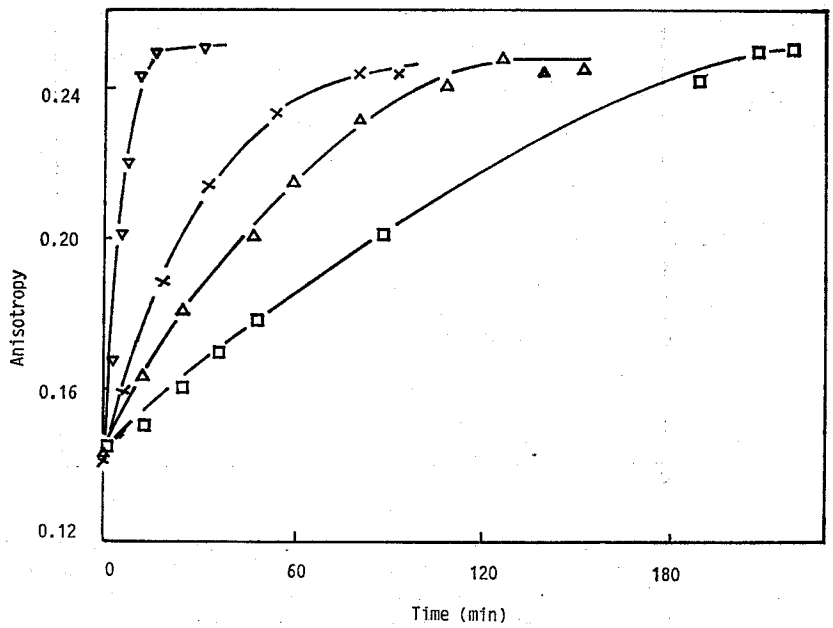

Fig. 3. Effects of oligomers on the rate of polymerization of AEDANS-actin monomer observed by the static anisotropy change. 0.5 mg/ml of AEDANS-actin in A-buffer was polymerized in 0.1 M KCl at 20°C. The concentration of oligomers added: (□); control, (△); 0.0009 mg/ml, (×); 0.0034 mg/ml, (▽); 0.0136 mg/ml

1.2 Effects of oligomers on the transformation of F-actin induced by EGTA

Miki et al. reported that the static anisotropy of F-actin increased upon EGTA addition.[7] Since G-actin to afford this change are not present in this solution, it is interesting to see what kind of changes in actin filaments corresponds to this anisotropy increase.

Oligomers were added to see if the parallel acceleration of the rate of anisotropy-with viscosity-increase after adding EGTA could be induced. Fig. 4 and Fig. 5 show that oligomers accelerate only the anisotropy increase but not the viscosity increase. This results indicate that oligomers do not work as nuclei in this case. Since if oligomers act as nuclei for plymerization, both of anisotropy and viscosity should increase as shown in section 1.1. The viscosity reached finally in the presence of oligomers is lower compared with that in their absence (Fig. 5). The most probable explanation for the effect of EGTA on F-actin in the presence of oligomers is the induction of network or bundle formation. The oligomer binding to F-actin seems to enhance the network formation. The lacking of the network formation under the viscometer may be due to the shear force which disturbs the binding of oligomers to filaments to some extent. The presence of some weak effect of oligomers are seen from the lower level of viscosity attained, compared with the control.

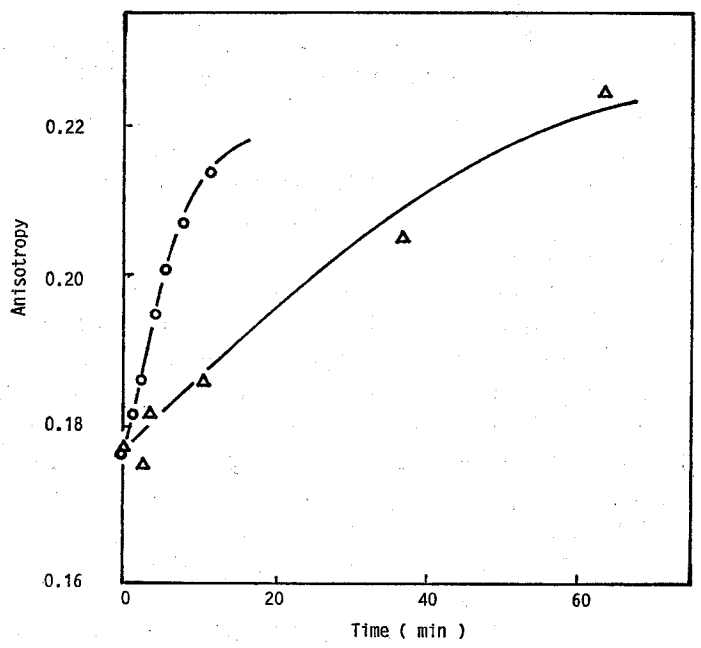

Fig. 4. Effects of oligomers on the rate of the EGTA-induced anisotropy increase of F-acitn. To 0.066 mg/ml of AEDANS-actin in 0.1 M KCl, 20 mM Tris-HCl (pH 8.0), 1 mM ATP, 1 mM 2-mercaptoethanol, 0.011 mM $CaCl_2$ and 1 mM NaN_3, EGTA was added to a concentration of 0.1 mM at zero time. (△); control, (○); 0.0085 mg/ml of oligomers

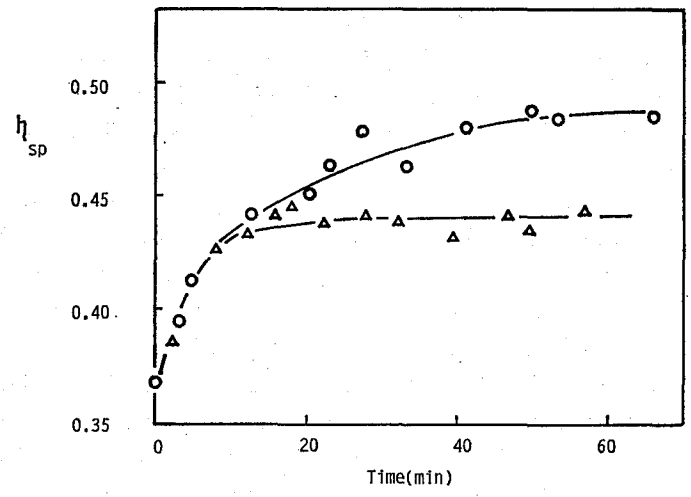

Fig. 5. Effects of oligomers on the viscosity change of F-actin induced by EGTA addition. To 0.1 mg/ml of AEDANS-actin in 0.1 M KCl, 20 mM Tris-HCl (pH 8.0), 1 mM ATP, 0.5 mM 2-mercaptoethanol, 0.01 mM $CaCl_2$, and 1 mM NaN_3, EGTA was added to a concentration of 0.1 mM at zero time. (○); control, (△); 0.0017 mg/ml of oligomers

2. The characteristics of Acto*S1 complex formed by EDC cross-linking

2.1 The change in Mg-ATPase activity of Acto*S1 according to depolymerization and reassembly

Mornet and associates[2] have shown that S1 and actin can be cross-linked with EDC, and the complex shows high Mg-ATPase activity. Sutoh[12] has demonstrated that actin and S1 can be cross-linked in a molar ratio of 1/1 and either 165 K or 175 K complex is formed according to the site of cross-linking on the heavy chain of S1. As reported in Ref. 3, the Acto*S1 filaments are depolymerized by dialysis at low ionic strength. Depolymerized Acto*S1 complex was purified with Ultrogel column (Fig. 6) and the fractions were observed with PAGE (Photo. 1). The first peak contains the depolymerized Acto*S1 complex and the second peak contains G-actin. As shown in Fig. 7, the Mg-ATPase activity of the depolymerized Acto*S1 is low, only 0.3 sec^{-1}. In contrast, there appeared considerable Mg-ATPase activity with incubation in the presence of excess actin monomers and 1 mM $MgCl_2$. Stoichiometric addition of actin monomers to the depolymerized Asto*S1 complex and following incubation in the presence of 1 mM $MgCl_2$ show the saturation of activity at a molar ratio of actin, approximately 400-fold greater than Acto*S1 complex (Fig. 8). The rate of the maximally enhanced Mg-ATPase activity with a saturation of actin was about 5.0 sec^{-1} which corresponds to 1/3–1/2 of the activity of the original undepolymerized Acto*S1 complex.

2.2 The change in K-EDTA ATPase activity of Acto*S1 according to depolymerization and reassembly

As described previously[13], the filamentous Acto*S1 complex loses its K-EDTA ATPase activity after depolymerization (Fig. 9). The ATPase activity recovers almost completely with a stoichimetric addition of actin monomers, approximately

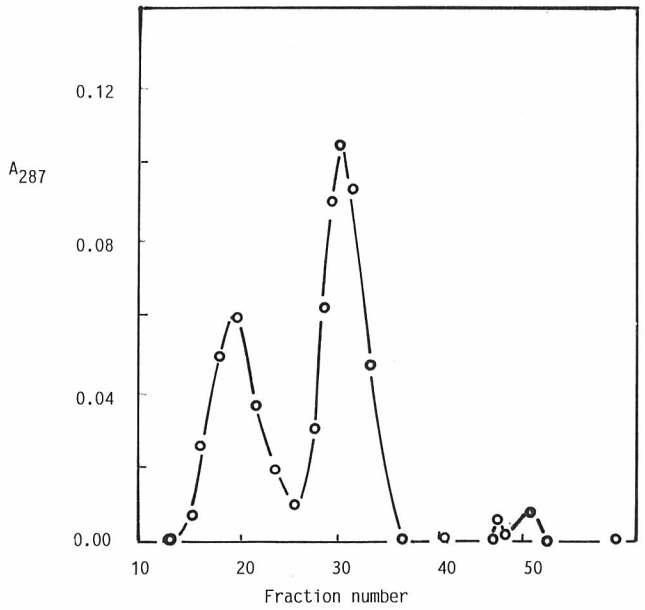

Fig. 6. Purification of the depolymerized Acto* Sl complex with AcA 34 Ultrogel (LKB) column (1.6 cm $\times$70 cm). 6 ml of Acto* S1 complex (1.0 mg/ml) in 0.2 mM ATP, 0.2 mM $CaCl_2$, 2 mM Tris-HCl Tris (pH 8.0) and 1 mM KN_3 was applied to the column and 2.4 ml/tube was collected.

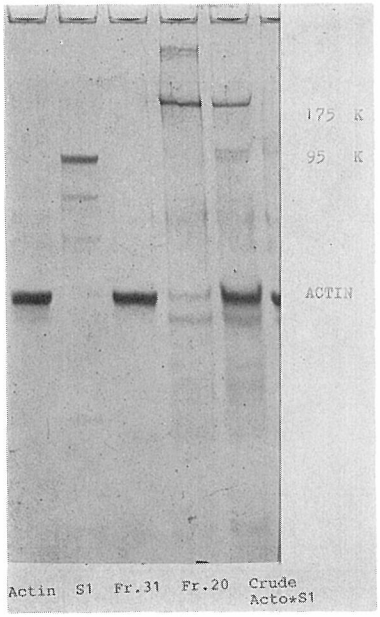

Photo. 1. SDS-polyacylamide gel electrophoresis of Acto* S1 complex obtained from the fraction shown in Fig. 6.

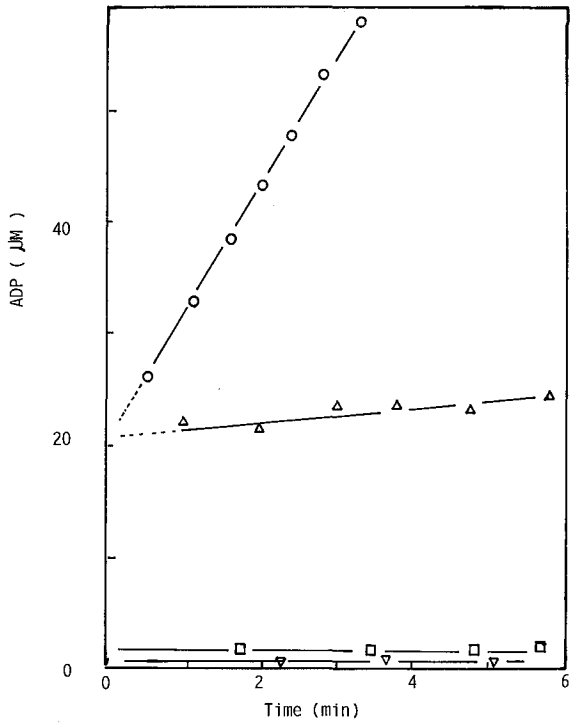

Fig. 7. The loss of the Mg-ATPase activity of depolymerized Acto* S1 complex (Fraction 20) and its recovery with incuvation in the presence of G-actin and 1 mM $MgCl_2$ for 1 hr. Mg-ATPase was measured with pyruvate kinase-LDH coupled assay system. For comparison, the absence of Mg-ATPase activity in Fr. 31 (G-actin) and Fr. 50 (light chain) after incuvation with G-actin in 1 mM $MgCl_2$ is shown. To 0.05 ml of each fraction, 0.005 ml of G-actin (6.7 mg/ml) was added. (△); Fr. 20, (○); Fr. 20+G-actin, (□); Fr. 31, (▽); Fr. 50

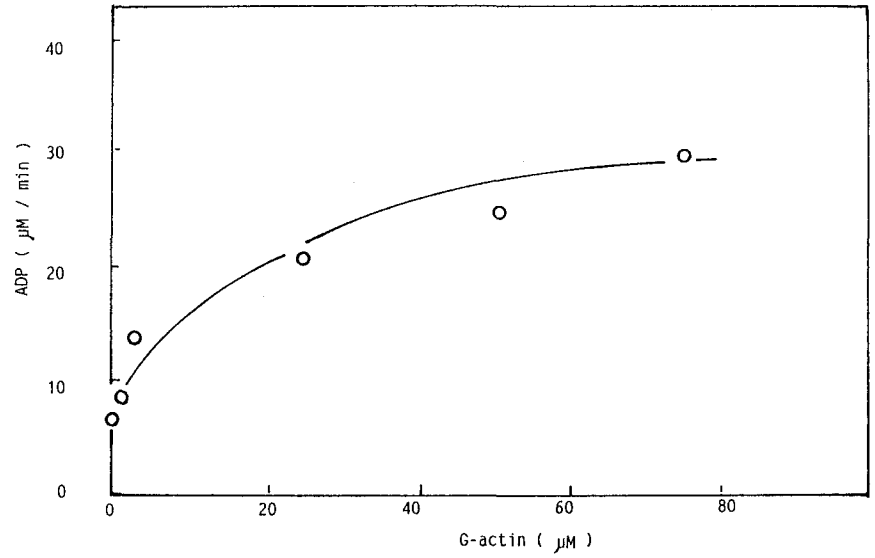

Fig. 8. The saturation of the Mg-ATPase activity of the depolymerized Acto* S1 (0.1 μM of cross-linked S1) complex by stoichiometric addition of G-actin. ATPase was measured after incuvation for 30 min in 1 mM $MgCl_2$.

100-fold greater than Acto*S1 complex and incuvation in 0.1 M KCl for 1 hr. Very interestingly, at the initial phase, this sample shows the decrease in the concentration of P_i once hydrolyzed enzymatically then the steady P_i liberation appears.

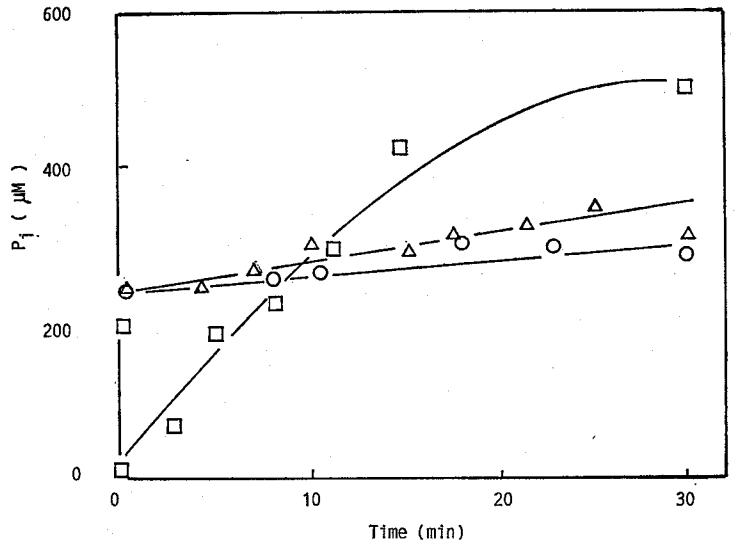

Fig. 9. The low K-EDTA ATPase activity of the depolymerized Acto* S1 complex and its enhancement with incuvation in the presence of G-actin and 0.1 M KCl, for 1 hr. Acto* S1 complexes (0.1 mg/ml of PIA-S1) in A-buffer were mixed with various amount of G-actin: (○); 0.04 mg/ml, (△); 0.2 mg/ml, (□); 2 mg/ml. ATPase activity was measured in 1 M KCl, 5 mM EDTA, and 50 mM Tris-HCl (pH 7.5), according to Ref. 6.

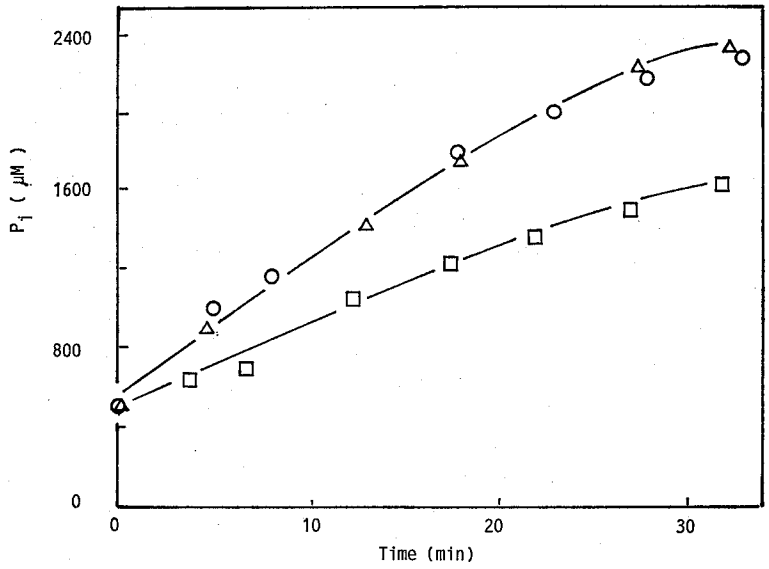

Fig. 10. The inverse effect of actin on the K-EDTA ATPase activity of S1 compared with Acto* S1. S1 (0.14 mg/ml of PIA-S1) was incuvated with G-actin: (○); control, (△); 0.2 mg/ml, (□); 2 mg/ml in 0.1 m KCl for 1 hr. ATPase was measured in 1 M KCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5).

Contrally to depolymerized Acto*S1, K-EDTA ATPase of labeled S1 is depressed with actin binding (Fig. 10).

DISCUSSION

Throughout this study, it has been undertaken to see the effect of small amount of cross-linked actin oligomers or Acto*S1 complexes on the properties of actin assembly. The cross-linked complexes were mixed with a large amount of G-actin and their assemblies were induced. The cross-linked oligomers accelerate the rate of actin assembly as seen by viscosity and anisotropy. Very early (1960), Ooi[11] suggested the dimer formation of G-actin, and later the presence of oligomers below the critical concentration was confirmed.[14-15] So as these oligomers which were formed without cross-linking, oligomers formed by cross-linking also accelerate the polymerization of G-actin with a formation of nuclei. Whereas contraly to oligomers which function as nuclei at the time of G-actin polymerization, the effect of oligomers on F-actin after EGTA addition seems rather peculiar, that is, the result of static anisotropy of fluorescence did not coincide with that of viscosity. In this case oligomers did not operate as nuclei. Instead oligomers may work as mediators for the network or bundle formation of filaments. If the binding of oligomers to F-actin is weak, shear force in viscometer may depress the effect of oligomers, although which is left partially as seen from the lower plateau of the viscosity attained in the presence of oligomers compared with the control. Miki and associates[7] have explained the EGTA effect on the anisotropy by a conformational change of F-actin. Present proposal may correspond to their assertion. There exist various proteins which affect the network formation of actin filaments.[16] Comparision of oligomers with these proteins seems interesting from the point of view of network formation. The study of the binding sites of oligomers will be one of the interesting future problems to clarify how oligomers make it possible to form networks. The rate of G-actin binding at the barbed end and pointed one is not same.[17-18] It is not clear yet which end is more affected by oligomer. Cytochalasin binds to the barbed end and there inhibits G-actin binding.[19] Cytochalasin also has another function to inhibit the network formation of actin.[18] Similarly, oligomers may have two functions.

At present the binding site of the depolymerized Acto*S1 to actin filaments is not clear. With an addition of excessive G-actin and incubation in the high ionic strength, its lost Mg-ATPase activity is recovered until about one half to one third of the initial value. However, the preliminary electron-microscopic study shows that the large amount of short particles are formed after the depolymerization and reassembly cycle of Acto*S1 complexes. This may suggest that the shape of actin assembled is controlled by a small amount of Acto*S1 introduced. As shown by the loss of ATPase activity, the cross-linking introduces strain into the proteins composing the complex, and the reassembly of the complex with actin releases the strain imposed on them. The key function of the actin filament seems to be transmissions of the conformational information from end to end.

As seen above, the characteristics of actin filaments are complicated and it is not

clear which phenomenon is really essential for the generation of muscle contraction. To overcome this barrier, we can not neglect the fact that evolution is responsible for the versatile characteristics of actin and myosin. Now it is well known that actin and myosin are not only proteins constituting muscles but also being responsible for other motility such as cytoplasmic streaming. Both proteins are extracted from the myxomycete *Physarum polycephalum*[20-21]. Although it may be the unsolved problem within a period as far as we can imagine that how actin and myosin became proteins playing a role for biological movement, but we should endeavor to understand the origin of biological movement and its evolution.

For the first step, it is essential to discriminate the biological movement from the Brownian motion. To see the translational Brownian motion, we must know the center of mass of micro particles. Therefore, the method to measure the center of mass was developed[22].

Furthermore, two important pursuites concerning the appearance of motility in the history of living organisms were executed. One is whether or not the conformational anisotropy of a molecule or a particle will produce the translational anisotropy of the Brownian motion[23] and another is how energy is coupled to these anisotropies to create the biological motility. According to this line, the studies of the streaming in the caffein drop obtained from the Plasmodia of *physarum polycephalum* were carried out.[24]

Even though we can not trace back the evolutional string, we are always strongly related to evolution. When we plan experiments concerned with the mechanism of motility, the questiones mentioned above remind us our trend that we conjecture that actin and myosin have acquired the most efficient conformation for energy transformation during evolution and remain usually in the conformation of the lowest free energy, therefore, such a perturbation as cross-linking to shift the distance of actin and S1 has increased the free energy of both proteins, and then extremely high ATPase activity was induced to recover the original conformation. In the line of this sense, the study of excimer of S1 labeled with a fluorescent probe is now in progress.[5]

Actin also has another properties to bind to proteins which are not essentially related to motility. For example binding with DNase 1 is a very important clue to find how actin have obtained such a characteristics and what is the function of it. The interaction of both proteins was studied with the time resolved fluorescence anisotropy measurement[20]. These kinds of researches may focus the light on the profound meaning hidden deeply in the conformation of muscle proteins.

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